AN ANTITHROMBOSIS ENZYME FROM THE SNAKE VENOM OF *AGKISTRODON ACUTUS*

RELATED APPLICATION

This application is converted from provisional application serial no. 60/043,886 filed April 10, 1997, the content of which is incorporated by reference herein in its entirety, including claims, sequences and drawings.

FIELD OF THE INVENTION

This invention relates to an antithrombosis enzyme derived from the snake venom of an *acutus* species.

BACKGROUND OF THE INVENTION

Anti-thrombus drugs extracted from *acutus* venom have been reported in the literature, e.g., "Preparation and Study of Anti-thrombus Enzymes No. 1, 2, and 3", Journal of the Medical Univ. of China, 1989.18 (special issue); and "Technique for Extracting Definriogenase from the venom of *Agkistrodon acutus*," CN 92102645.5 (CN 1065680.A). These anti-thrombus drugs are proteinase components extracted from the snake venom. They act

like thrombase with hemorrhagic side-effect. In addition, some of these products are not single component proteinase, but a mixture of different components, which limits the pharmaceutical application of these drugs in human.

Other snake venom derived pharmaceutical products include Ancrod, Trigtamin and Integrilin (*see* Matsuzaki et al., Biochem. Biophy. Res. Com. 220(2):382-387, 1996; Morita et al., Natural Toxins II, pp187-196, Edited by B.R. Singh and A.T. Tu, Plenum Press, New York, 1996; U.S. Patents 5,196,403, 5,242,810, 5,453,370, 4,017,012, 5,344,783, 5,686,571, 5,523,292, 5,066,592 and 5,342,830).

SUMMARY OF THE INVENTION

Within the scope of this invention, Applicant has extracted, purified and cloned an antithrombosis enzyme (ATE, also called a fibrinolytic enzyme in the provisional application) from the venom of Southern-Anhui *Agkistrodon acutus* in China. This enzyme degrades both fibrinogen and fibrin, and inhibits platelet aggregation. It is useful for preventing and treating vaso-occulusive and thromboembolic disorders, including, but not

limited to, myocardial infarction, restenosis, peripheral anginaphraxis, angiopathic thrombosis, cerebral thrombosis, ischemic cerebral vascular diseases, unstable angina, acute thrombosis, unstable stenocardia and hemiparalysis caused by cerebral thrombosis.

The present invention provides methods and compositions for preventing or treating diseases and processes mediated (caused or aggravated) by undesired and/or uncontrolled thrombosis by administering to a human or animal a composition containing or capable of expressing the antithrombosis enzyme in a dosage sufficient to prevent, reduce, eliminate or inhibit thrombosis. The antithrombosis enzyme may be substantially purified or in a crude extract. The antithrombosis enzyme may be produced from snake venom, chemically synthesized or expressed from a recombinant vector. It may also be combined with a pharmaceutically acceptable excipient or carrier, and optionally sustained-release compounds or compositions, such as biodegradable polymers, to form therapeutic compositions.

The present invention is particularly useful for treating or preventing acute and recurrent cerebral thrombosis,

myocardial infarction, restenosis, peripheral anginaphraxis, angiopathic thrombosis, ischemic cerebral vascular thrombosis, unstable angina, unstable stenocardia, and thromboangitis obliterans. Administration of the antithrombosisi enzyme can prevent blood clot formation and reduce, diminish or dissolve blood clot. The antithrombosis enzyme may also be used in combination with other compositions and procedures for the treatment of thrombosis. For example, it may be used in combination with a thrombolytic agent known in the art, which includes, but is not limited to, tissue plasminogen activator purified from natural sources, recombinant tissue plasminogen activator, streptokinase, urokinase, prourokinase, anisolated streptokinase plasminogen activator complex (ASPAC), animal salivary gland plasminogen activators and known, biologically active derivatives of any of the above. In these combination compositions, the antithrombosis enzyme and other thrombolytic agent work in a complementary fashion to dissolve blood clots, resulting in decreased reperfusion times and increased reocclusion times in patients treated with them. The use of the antithrombosis enzyme in the compositions of this invention

advantageously allows the administration of a thrombolytic reagent in dosages previously considered too low to result in thrombolytic effects if given alone. This avoids some of the undesirable side effects associated with the use of thrombolytic agents, such as bleeding complications. The compositions of this invention may also be used before, concurrent with, or after angioplastic or fibrolytic treatment to prevent or treat restenosis.

Thus, in a first aspect, this invention features an isolated, purified or recombinant antithrombosisi enzyme which has (i) a molecular weight of between about 28 kD and about 32 kD when analyzed by polyacrylamide gel electrophoresis, (ii) an aspartic acid content of between about 2% and about 5%, and (iii) a glutamic acid content of between about 2% and about 5%. This enzyme has the ability to hydrolyze fibrin, dissolve thrombus, inhibit platelet aggregation, and inhibit the formation of thrombus.

In a preferred embodiment, the enzyme has fibrinolytic activity of no less than one fibrinolytic activity unit per mg protein. In another preferred embodiment, the enzyme has

fibrinolytic activity of between about one and about three fibrinolytic activity units per mg protein. This enzyme specifically hydrolyzes the A (α) chain of fibrinogen. This enzyme completely or almost completely inhibits human platelet aggregation induced by agonists such as ADP, Epinephrine, Thrombin and collagen. This enzyme has no detectable hydrolysis effect on casein. The enzyme dissolves arterial and venous thrombus in a mammal, prevent thrombosis, reduce blood viscosity, and improve microcirculation. At the same time, this enzyme has minimum effect on the thromosystem, resulting in little possibility of hemorrhage. This enzyme is different from related enzymes from other *Acutus* species (e.g., IX/X binding proteins, Matsuzaki et al., Biochem. Biophy. Res. Com. 220(2):382-387, 1996; Morita et al., Natural Toxins II, pp187-196, Edited by B.R. Singh and A.T. Tu, Plenum Press, New York, 1996) in that this enzyme has both fibrinolytic activity and antiplatelet aggregation activity, and less hemorrhagic activity.

In other preferred embodiments, this enzyme is purified from Southern-Anhui *Agkistrodon acutus*. The enzyme is a heterodimer of A chain and B chain each with a molecular weight

of about 14 KD to about 16 KD. The A chain has at its amino end the following sequence:

Asp-Cys-Ser-Ser-Asp-Trp-Ser-Ser-Tyr-Glu-Gly-His-Cys-Tyr-Lys-Val-Phe-Lys-Gln-Ser-Lys-Thr-Trp-Thr-Asp-Ala-Glu-Ser-Phe-, and the B chain has at its amino end the following sequence:

Asp-Cys-Pro-Ser-Glu-Trp-Ser-Ser-Tyr-Glu-Gly-Phe-Cys-Tyr-Lys-Pro-Phe-. Preferrably, the A chain and the B chain are linked by one or more disulfide bond.

In other preferred embodiments, this antithrombosis enzyme contains Ca^{++} and/or has aspartic acid at its amino terminus.

By "isolated" in reference to a polypeptide is meant a polypeptide isolated from a natural source or synthesized. The isolated polypeptides of the present invention are unique in the sense that they are not found in a pure or separated state in nature. Use of the term "isolated" indicates that a naturally occurring amino acid sequence has been removed from its normal cellular environment. Thus, the sequence may be in a cell-free solution or placed in a different cellular environment. The term does not imply that the sequence is the only amino acid chain

present, but that it is the predominate sequence present (at least 10 - 20% more than any other sequence) and is essentially free (about 90 - 95% pure at least) of non-amino acid material naturally associated with it.

By "enriched" in reference to a polypeptide is meant that the specific amino acid sequence constitutes a significantly higher fraction (2 - 5 fold) of the total of amino acids present in the cells or solution of interest than in normal or diseased cells or in the cells from which the sequence was taken. This could be caused by a person by preferential reduction in the amount of other amino acids present, or by a preferential increase in the amount of the specific amino acid sequence of interest, or by a combination of the two. However, it should be noted that enriched does not imply that there are no other amino acid sequences present, just that the relative amount of the sequence of interest has been significantly increased. The term "significantly" here is used to indicate that the level of increase is useful to the person making such an increase, and generally means an increase relative to other amino acids of about at least 2 fold, more preferably at least 5 to 10 fold or

even more. The term also does not imply that there is no amino acid from other sources. The amino acid from other sources may, for example, comprise amino acid encoded by a yeast or bacterial genome, or a cloning vector such as pUC19. The term is meant to cover only those situations in which man has intervened to elevate the proportion of the desired amino acid.

By "purified" in reference to a polypeptide does not require absolute purity (such as a homogeneous preparation); instead, it represents an indication that the sequence is relatively purer than in the natural environment (compared to the natural level this level should be at least 2-5 fold greater, *e.g.*, in terms of mg/ml). Purification of at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated. The substance is preferably free of contamination at a functionally significant level, for example 90%, 95%, or 99% pure.

By "recombinant" is meant a polypeptide or enzyme produced by recombinant DNA techniques such that it is distinct from a naturally occurring polypeptide either in its location (e.g., present in a different cell or tissue than found in

nature), purity or structure. Generally, such a recombinant polypeptide will be present in a cell in an amount different from that normally observed in nature. This invention features recombinant ATE and its fragments obtainable using techniques known to those skilled in the art, including those described in McDonnell et al., U.S. Patent Application No. 08/223,943 filed April 6, 1994, Evans et al., U.S. Patent 5,071,773, and PCT application, PCT/US91/00399 filed January 22, 1991 (International Publication No. WO 91/12258), incorporated by reference herein.

In a second aspect, this invention features isolated, purified or recombinant polypeptide fragments of the A chain and the B chain of the antithrombosis enzyme. Preferably, these fragments contain no less than 15, 20, 30 or 40 contiguous amino acid residues from the A or B chain. For example, these fragments may contain no less than 15, 20, 30 or 40 contiguous amino acid residues from SEQ ID NO: 2. Such polypeptide fragments can be synthesized chemically or expressed from recombinant vectors. They are useful for generating monoclonal antibodies which bind to both the polypeptide fragments and the intact antithrombosis enzyme (*see* U.S. Patents 5,733,738,

5,015,571, incorporated by reference herein). Monoclonal antibodies so generated can be attached to solid support and used to purify the antithrombosis enzyme from crude venom extract or cell extract by affinity chromatography.

The recombinant polypeptide fragments of the A chain and the B chain can be expressed from recombinant nucleic acid encoding such polypeptide fragments. For example, polypeptide fragments of the A chain can be expressed from recombinant nucleic acid containing no less than 45, 60, 90 or 120 contiguous nucleotides from SEQ ID NO: 1 or its fully complementary strand of the same length and a promoter effective to initiate transcription of the contiguous nucleotides in a host cell.

In yet another aspect the invention features an isolated, enriched, or purified antibody (e.g., a monoclonal or polyclonal antibody) having specific binding affinity to the antithrombosis enzyme or a fragment thereof. The antibody contains a sequence of amino acids that is able to specifically bind to the antithrombosis enzyme. The antibody may be prepared with techniques known to those skilled in the art, including, but not limited to, those disclosed in Niman, PCT application

PCT/US88/03921 (International Publication No. WO 89/04489), incorporated by reference herein. By "specific binding affinity" is meant that the antibody will bind to the ATE in a certain detectable amount but will not bind other polypeptides to the same extent under identical conditions.

In another aspect the invention features a hybridoma which produces an antibody having specific binding affinity to the antithrombosis enzyme or a fragment thereof. By "hybridoma" is meant an immortalized cell line which is capable of secreting an antibody.

In another aspect, the invention features an isolated, purified, enriched or purified recombinant nucleic acid encoding the antithrombosis enzyme, a chain of the enzyme, or fragments of the A chain or B chain. For example, the recombinant nucleic acid contains a sequence contiguously encoding SEQ ID NO: 2 and a promoter effective to initiate transcription of the conding sequence in a host cell. In particular, the recombinant nucleic acid contains SEQ ID NO: 1 operably linked to a promoter.

By "isolated" in reference to nucleic acid is meant DNA or RNA isolated from a natural source or synthesized. The